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Comparison of Two Target Genes for Detection and Genotyping of *Giardia lamblia* in Human Feces by PCR and PCR-Restriction Fragment Length Polymorphism

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A PCR assay targeting the *tpi* gene was developed to detect and to genotype *Giardia lamblia* in human feces. Our assay was specific and discriminated between *G. lamblia* assemblages A and B. *G. lamblia* cysts isolated from human feces were also analyzed with two previously described PCR-restriction fragment length polymorphism (RFLP) assays, which are based on the detection of *tpi* or *gdh* genes. These RFLP analyses distinguished groups I and II within assemblage A or groups III and IV within assemblage B. Among 26 fecal samples from patients with sporadic giardiasis diagnosed by hospital laboratories, the *tpi* gene was amplified from 25 (96%) with our PCR assay, whereas only 21 (81%) samples were positive when the *gdh* gene was targeted. Of the 25 positive samples, nine (36%) contained assemblage A and 16 (64%) contained assemblage B. Thus, RFLP analysis classified eight samples (32%) in assemblage A group II, eight (32%) in assemblage B group III, and five (20%) in assemblage B group IV. The group could not be specified for four samples. The *tpi* and *gdh* genes of *G. lamblia* assemblage B were amplified from 14 (93%) of 15 samples collected only from French soldiers coming back from the Ivory Coast. All of these contained assemblage B group III. The PCR method developed is sensitive, simple, and specific and shows that the *tpi* gene is well adapted for *G. lamblia* genotyping.

The intestinal protozoan *Giardia lamblia* (synonyms, *G. intestinalis* and *G. duodenalis* [1]) is a cosmopolitan parasite frequently involved in human parasitic gastroenteritis throughout the world. Transmission of the *G. lamblia* cyst to humans occurs mainly following ingestion of contaminated water. Clinical manifestations of symptomatic giardiasis include greasy stools, flatulence, diarrhea, and abdominal cramps (9). However, the majority of cases are asymptomatic or minimally symptomatic in immunocompetent individuals.

Among the six species identified in the *Giardia* genus, only *G. lamblia* infects humans and numerous other mammals as well (1, 25). Moreover, isolates of *G. lamblia* are classified into seven assemblages, based on the characterization of the glutamate dehydrogenase (*gdh*), small-subunit (SSU) rRNA, and triosephosphate isomerase (*tpi*) genes (12, 18, 20, 21). Assemblages A and B infect humans and a broad range of other hosts, including livestock, cats, dogs, and wild mammals. The assemblage A isolates have been further grouped into subgroups I and II. The assemblage B isolates have been separated into subgroups III and IV (17, 24). Genetic assemblages C, D, E, F, and G appear to be host restricted to domestic animals, livestock, and wild animals (19, 21).

At present, antigen detection immunoassays for *Giardia* are used as the routine diagnostic procedure of choice in many hospitals and public health laboratories (8, 13, 27). However, these methods are unable to differentiate between the genetic asemblages of *Giardia lamblia*. Molecular detection methods based on PCR have been developed to detect *G. lamblia* cysts in feces. These techniques have numerous advantages in terms of sensitivity, speed, and specificity in comparison to conventional methodologies (3, 16). Moreover, these molecular techniques may allow the genotyping of *Giardia lamblia* cysts (3, 4, 20).

We previously described a method for the successful extraction and detection of giardial DNA from naturally contaminated wastewater (5). In the present study, we evaluated primers for rapid and sensitive classification of *G. lamblia* cysts from human feces into assemblages A and B. These primers were previously designed for the detection and quantification of *G. lamblia* assemblages A and B in environmental samples by real-time PCR (data not shown). In this study, we evaluated the distribution of these major assemblages in sporadic human giardiasis in France and also in samples from 15 French soldiers coming back from the Ivory Coast. Moreover, the simultaneous use of our assay and two previously described PCR-restriction fragment length polymorphism (RFLP) assays allowed comparison of the *tpi* and *gdh* genes for detection and genotyping of *G. lamblia.*

MATERIALS AND METHODS

Purified suspensions. *Giardia lamblia* assemblage B cysts, produced by passage of human strain H3 (10) of *G. lamblia* through Mongolian gerbils, were obtained from Waterborne Inc. (New Orleans, La.). Prior to delivery, cysts were 95 to 99% purified by sucrose and Percoll density gradient centrifugation and washing with water, and then stored in phosphate-buffered saline (PBS, pH 7.4) with antibiotics. *Giardia lamblia* (assemblage A) cysts were purified from one human feces sample by the ethyl acetate procedure (see below) followed by

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TABLE 1. Results of PCR and PCR-RFLP analyses of the *Giardia lamblia tpi* and *gdh* genes amplified from 26 samples corresponding to sporadic cases

Hospital (no. of samples)	Sex[a]	Age (yr)	PCR[d]	*tpi* PCR[e]	RsaI[b]	GDHiF-GDHiR	NlaIV[b]	RsaI	Final result
CHU Brabois, Nancy (n = 11)									
	M	54	B	B		+	B	B-IV	B-IV
	F	1	B	B		+	B	B-IV	B-IV
	M	34	B	B		+	B	B-IV	B-IV
	M	1	B	B		+	B	B-III	B-III
	M	39	A	A[c]	A-II	—[c]			A-II
	M	21	A	A[c]	A-II	+	A-II		A-II
	M	50	A	A[c]	A-II	+	A-II		A-II
	F	37	B	B[c]		+[c]	B	B-III	B-III
	M	1	A	A[c]	A-II	+	A-II		A-II
	M	33	A	A[c]	—	+	A-II		A-II
	M	29	A	A	A-II	+	A-II		A-II
Pitie-Salpetriere Hospital, Paris (n = 6)									
	M	38	B	B		+	B	B-III	B-III
	NA	NA	—	—[c]		—[c]			Negative
	M	61	B	B		+	B	B-III	B-III
	F	<1	B	B		+	B	B-III	B-IV
	M	<1	B	—[c]		—[c]			B
	M	2	A	A	A-II	+	A-II		A-II
Bichat Hospital, Paris (n = 1)									
	M	37	B	B		+[c]	—		B
Legouest Army Hospital, Metz (n = 8)									
	NA	>18	A	A[c]	—	—[c]			A
	NA	>18	B	B		+	B	B-III	B-III
	NA	>18	B	B		—[c]			B
	NA	>18	B	B		+	B	B-III	B-III
	NA	>18	B	B		+	B	B-IV	B-IV
	NA	>18	A	A	A-II	+	A-II		A-II
	F	22	B	B		+	B	B-III	B-III
	M	24	B	B		+	B	B-III	B-III

[a] M, male; F, female; NA, not available.
[b] —, insufficient quantity of PCR product for RFLP analysis.
[c] DNA amplification by double PCR.
[d] A-PCR and B-PCR. —, negative results.
[e] TPIA-PCR and TPIB-PCR.

Percoll/sucrose flotation at a 1:10 dilution and a sucrose density gradient (7). Purified *Giardia muris* cysts of the Roberts-Thompson isolate were obtained from Waterborne Inc.

DNA samples. Three DNA samples of *Giardia lamblia* from cattle, sheep, and pigs were included in this study. A sequencing analysis had showed 100% matches between the amplified products (165 bp) obtained with these samples and the sequence with GenBank accession number AF069559 corresponding to *G. lamblia* assemblage E strain P-15 (data not shown). DNA recovered from *Cryptosporidium parvum* (Institut National de la Recherche Agronomique, Nouzilly, France), *Entamoeba histolytica*, *Entamoeba dispar* (Institut de Parasitologie, Strasbourg, France), *Campylobacter jejuni* (ATCC 29428; Central Hospital, Nancy, France), *Salmonella enterica* Serovar Typhimurium WG49, and *Escherichia coli* K12 Hfr was also included in this study.

Fecal samples. Forty-one fecal samples from patients in which *Giardia* cysts had been detected by conventional techniques were collected during this study; 26 fecal samples originated from patients with sporadic cases of giardiasis diagnosed between October 2000 and September 2004. Of these, 18 samples were provided by three civilian hospitals (Centre Hospitalier Universitaire Brabois, Nancy; Pitié-Salpêtrière Hospital, Paris; and Bichat Hospital, Paris, France) and eight samples from adults were sent by a military hospital (Legouest Army Hospital, Metz, France). Of the 18 patients in civilian hospitals, 61% were adults aged between 21 to 61 years and 78% were males (Table 1). No information about the immune status of these patients was provided by the laboratories. Fifteen samples from French soldiers (males aged between 23 and 50 years) were also collected from Legouest Army Hospital. These 15 cases of giardiasis were diagnosed in April 2004 after a 4-month stay in the same city in Ivory Coast (West Africa).

Cyst purification from fecal samples. Cysts were purified either by gel filtration chromatography as previously described (23) or by the ethyl acetate procedure adapted from the formalin ethyl acetate method (26). The ethyl acetate procedure was performed with a maximum of 5 g feces suspended in 20 ml deionized water and 6 ml ethyl acetate.

Microscopy. The staining procedure was carried out as previously described (5).

DNA extraction. All buffers and reagents used in this step are provided in the QIAamp DNA stool kit (QIAGEN, Courtaboeuf, France), except buffer AVL and RNA carrier (Qiagen). The DNA extraction was carried out on a 200-μl purified sample. Three modifications of this protocol were employed as previously described (5). (i) Cyst wall lysis and adsorption of impurities were improved by increasing the time of incubation at 95°C to 10 min and the time of incubation with InhibitEx to 3 min; (ii) 5 μl RNA carrier (1 μg/μl in buffer AVL) was added during the protein digestion step; and (iii) protein digestion and column purification were applied twice per sample. After the first elution of DNA extract from the column, the protocol was started again at the protein digestion step. The DNA was then stored at −80°C.

PCR amplification and restriction fragment length polymorphism analysis. (i) Oligonucleotide primers. Five sets of oligonucleotide primers obtained from Proligo France were used for the analysis of fecal samples. Two sets of primers for detection of *Giardia lamblia* assemblages A and B were designed against the coding region of the *tpi* gene using the Primer Express Oligo Design software (v. 1.5; Applied Biosystems). ClustalX (v. 1.8) was used for the determination of the variations of sequence between the following *Giardia* species and genotypes (GenBank accession number): *G. lamblia* assemblage A: WB (L02120), JH (U57897), Ad-1 (AF069556), Ad-2 (AF069557), isolate 2907 (AY228647), isolate from wild deer (AY302562); assemblage B: GS/M (L02116), BAH-12

(AF069561), Ad-19 (AF069560), isolate 2924 (AY228628), isolate 2582 (AY228629), isolate 2506 (AY228630), isolate 2887 (AY228631), isolate 2902 (AY228632), isolate 2877 (AY228633), isolate 2900 (AY228634), isolate 2901 (AY228635), isolate 3470 (AY228636), isolate 3565 (AY228637), isolate 3577 (AY228638), isolate 1758 (AY228639); assemblage C: Ad-136 (AF069563), isolate 2643 (AY228641), isolate 2669 (AY228642), isolate 2674 (AY228643), isolate 2665 (AY228644); assemblage E: P-15 (AF069559), isolate 109 (AY228645), isolate 15 (AY228646); assemblage F: Ad-23 (AF069558); and assemblage G: Ad-157 (AF069562), isolate 2135 (AY228640); *G. ardeae* (AF069562); *G. microti*: isolate 3463 (AY228648), isolate 3460 (AY228649); *G. muris* (AF069565).

The primers used for assemblage A amplification were forward (A-for) 5′-GGAGACCGACGAGCAAAGC-3′ (positions 839 to 857 on the WB sequence, GenBank no. L02120), and reverse (A-rev), 5′-CTTGCCAAGCGCCTCAA-3′ (positions 970 to 986 on the WB sequence). A 148-bp fragment of the assemblage A gene was amplified with primers A-for and A-rev (A-PCR). The primers used for assemblage B amplification were forward (B-for), 5′-AATAGCAGCACA RAACGTGTATCTG-3′ (positions 126 to 150 on the BAH-12 sequence, GenBank no. AF069561), and reverse (B-rev), 5′-CCCATGTCCAGCAGCATC T-3′ (positions 188 to 206 on the BAH-12 sequence). An 81-bp fragment of assemblage B gene was obtained with primers B-for and B-rev (B-PCR). Primers sets TPIAF-TPIAR (TPIA-PCR) and TPIBF-TPIBR (TPIB-PCR) were used as previously described (2) for amplification of the *G. lamblia tpi* gene from assemblages A and B, respectively. Finally, primer set GDHiF-GDHiR was used for amplification of the *G. lamblia gdh* gene, as previously described (20).

(ii) PCR amplification and RFLP analysis. Amplification of the *tpi* and *gdh* genes was performed as a single PCR. Amplification reactions (50 μl) contained 5 μl of DNA, 1× PCR buffer corresponding to a final concentration of 1.5 mM $MgCl_2$ (Qiagen), each deoxynucleotide triphosphate at a concentration of 200 μM (Applied Biosystems), each primer at a concentration of 0.5 μM, and 2.5 U of HotStarTaq DNA polymerase (Qiagen). Cycling parameters were 15 min at 95°C (initial heat activation step), followed by 50 cycles of 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C, with a final extension of 7 min at 72°C. Both positive and negative controls were included in each PCR to validate results. Quantities of DNA equivalent to 600 *G. lamblia* assemblage A (purified from feces) cysts and 4,500 *G. lamblia* assemblage B (strain H-3) cysts were used as the templates for the positive controls, and distilled water was used as the template for negative controls throughout.

RFLP analysis was performed by digesting 2.5 μl of PCR product with 2 U of RsaI (Promega) or 2 U of NlaIV (New England Biolabs) in 1× enzyme buffer in a final volume of 20 μl for 3 h at 37°C. The RsaI digestion allowed the distinction between assemblage A group I and group II after amplification with the TPIAF and TPIAR primers (2). The NlaIV digestion was used for the distinction between assemblage A group I, assemblage A group II, and assemblage B after amplification with the GDHiF and GDHiR primers. RsaI digestion distinguished between assemblage B group III and assemblage B group IV after use of the GDHiF and GDHiR primers (20).

(iii) PCR product and restriction fragment detection. PCR products and restriction fragments were separated by horizontal electrophoresis in 2 and 3.2% agarose gels, respectively, with ethidium bromide (0.6 μg/ml) staining. A 100-bp DNA ladder (Promega) was included as a size marker. PCR products and restriction fragments were recorded by UV transillumination.

RESULTS

First, the genotype of *Giardia lamblia* cysts from one fecal sample classified in assemblage A with TPIA-PCR and TPIB-PCR (2) was reconfirmed by sequencing analysis. This analysis showed 100% matches between the amplified product obtained with TPIA-PCR (577 bp) and the sequence with GenBank accession number U57897 corresponding to *G. lamblia* assemblage A group II. The sensitivity of A-PCR and the specificity of both A-PCR and B-PCR could be evaluated with this purified suspension of cysts.

The specificity of our PCR assay was first evaluated by subjecting the primers to a BLAST test (http://www.ncbi.nlm.nih.gov/BLAST/). For the A-for and A-rev primers, the BLAST tests returned sequences other than *G. lamblia* assemblage A, and only one sequence showed a 100% match with the A-rev primer. However, the sequences other than *G. lamblia* assemblage A returned by these two BLAST tests were different. For the B-for and B-rev primers, the BLAST tests returned sequences other than *G. lamblia* assemblage B with one to eight mismatches. Furthermore, the mismatched sequences returned by these two BLAST tests were different.

The specificity was then examined by performing PCR assays. By using a quantity of DNA equivalent to 600 *Giardia lamblia* assemblage A cysts per reaction, the predicted 148-bp product was obtained by A-PCR, but no product was amplified by B-PCR. Conversely, by using a quantity of DNA equivalent to 4,500 *Giardia lamblia* assemblage B (strain H3) cysts per reaction, the predicted 81-bp product was observed with B-PCR, but no product was obtained with A-PCR. No product was amplified by performing either A-PCR or B-PCR with DNA extracted from purified *Giardia lamblia* assemblage E from cattle, sheep, and pigs, *Giardia muris*, *Cryptosporidium parvum*, *Entamoeba histolytica*, *Entamoeba dispar*, *Campylobacter jejuni*, *Salmonella enterica*, or *Escherichia coli*.

To estimate the sensitivities of our PCR assays, 10-fold dilutions of purified cysts were performed in PBS (pH 7.2) prior to DNA extraction. The amplification step was then performed in triplicate on 10-fold serial dilutions of cysts. For *G. lamblia* assemblage A, the predicted 148-bp product was obtained for each well of the triplicates for concentrations ranging from 600 to 6 cysts per reaction mixture. For *G. lamblia* assemblage B, the 81-bp product was observed for each well of the triplicates for concentrations ranging from 4,500 to 4.5 cysts per reaction mixture.

Among the 26 fecal samples corresponding to sporadic giardiasis diagnosed in hospital laboratories, the presence of *Giardia* cysts was reconfirmed by immunofluorescence (IF) microscopy for all of these during our study. On the other hand, the number of positive samples by PCR varied according to the PCR assay employed and the target gene amplified. Indeed, the *tpi* gene was amplified from 25 samples (96.1%) with A-PCR and B-PCR developed in our laboratory. The same target gene was amplified from 24 samples (92.3%) with TPIA-PCR and TPIB-PCR. However, only 18 positive samples (69.2%) were obtained with a single PCR. A double PCR allowed the achievement of six additional positive samples. For the *gdh* gene, 21 samples (80.7%) were positive with the GDHiF and GDHiR primers. Moreover, a single PCR resulted in 19 positive samples (73.1%) and a double PCR was needed for two other samples.

Regarding classification into assemblages A and B (Table 1), the results of the various PCR and PCR- RFLP methods were in agreement for the sporadic cases. Assemblage A was detected in nine (34.6%) and seven (26.9%) samples by using the *tpi* and *gdh* genes as the target, respectively. When targeting the *tpi* gene, assemblage B was identified in 16 (61.5%) and 15 (57.6%) samples with B-PCR and TPIB-PCR, respectively. However, when using the *gdh* gene as the target, assemblage B was detected in only 13 (50%) fecal samples.

A more specific analysis of these sporadic cases was then conducted with the distinction between assemblage A groups I and II and assemblage B groups III and IV on the basis of their fragment patterns obtained by RFLP analysis (Table 1). The results obtained with the two published methods were in agreement for sporadic cases. Whatever the gene used, subgroup I was never observed in these fecal samples. On the other hand,

TABLE 2. Summary of genotyping results obtained with the three methods

Samples	Assemblage and group	No. of samples identified			
		A-PCR and B-PCR	Amar et al. (2)	Read et al. (20)	Final results
Sporadic cases (n = 26)					
	A	9	9	7	9
	Group II		7	7	8
	Group not determined		2	0	1
	B	16	15	13	16
	Group III			8	8
	Group IV			5	5
	Group not determined				3
French soldiers (n = 15)					
	A	0	0	0	0
	B	14	14	14	14
	Group III			14	14

six samples were genotyped as assemblage A group II with both RFLP techniques and two samples were classified in this group with only one of these methods. For one sample classified in assemblage A by PCR assays targeting the *tpi* gene, the group could not be specified. Indeed, the quantity of PCR product obtained with A-PCR was insufficient for RFLP analysis, and no product was amplified with the GDHiF and GDHiR primers.

Only one method used in our study allowed distinction between assemblage B groups III and IV (20). The RFLP analysis resulted in eight and five samples classified in assemblage B group III and group IV, respectively. For three samples identified as assemblage B with our PCR assay, the group could not be specified with the method described by Read and colleagues (20). Of these, two samples were negative after double PCR with the GDHiF and GDHiR primers. For the third sample, the quantity of PCR product obtained with the GDHiF and GDHiR primers was insufficient for RFLP analysis. The genotyping results are summarized in Table 2.

Finally, 31% of the samples were classified in assemblage A group II and in assemblage B group III as well. Assemblage B group IV was detected in 19% of the samples. The *G. lamblia* cysts used for validation of our A-PCR assay were classified in assemblage A group II by the RFLP analysis, as they were by the sequencing analysis carried out at the beginning of our study.

Among the 15 cases of giardiasis diagnosed in samples from French soldiers coming back from the Ivory Coast, the presence of *Giardia* cysts was reconfirmed by IF microscopy for 100% of the samples. The *Giardia lamblia* assemblage B genotype was isolated in 93.3% of the samples with the three methods, and *tpi* or *gdh* gene fragments were not amplified from only one sample. Moreover, the PCR-RFLP analysis developed by Read and colleagues (20) allowed the detection of assemblage B group III in all of these samples (Table 2).

DISCUSSION

In the present study, we developed a conventional PCR assay allowing the rapid detection and distinction of *G. lamblia* assemblages A and B in human feces. When applied to 10-fold serial dilutions of purified *G. lamblia* cysts, this PCR assay showed a high degree of sensitivity. Thus, a threshold of 6 and 4.5 purified cysts per reaction was reached by single PCR for assemblages A and B, respectively. Moreover, our primers were validated by the comparison between the genotyping results obtained with our PCR assay and previously described PCR-RFLP methods (2, 20). For sporadic cases of giardiasis confirmed by IF analysis (n = 26), our PCR resulted in 96% positive samples with a single amplification step. The negative results observed for 4% of the samples could be explained by the presence of parasites at a very low level or degradation of parasite DNA during transport of the samples to our laboratory. The amplification of these 26 samples with the primers described by Amar and colleagues (2) and Read and colleagues (20) allowed us to obtain 92% and 81% positive samples, respectively. However, double amplification was then needed for six and two samples, respectively, with these previously described methods.

In the study conducted by Amar and colleagues (2), amplification of the *tpi* gene was performed as a two-step PCR (seminested PCR) and resulted in 94% positive samples (n = 35). The ethyl acetate procedure, which is adapted from the formalin ethyl acetate method, was tested for fecal samples. This method allowed rapid and efficient purification of *Giardia* cysts prior to DNA extraction. Indeed, at least 15 fecal samples were simultaneously purified with this method, whereas the gel filtration chromatography method was time-consuming and limited to four samples per purification step. The ethyl acetate procedure is well adapted for the purification of *Giardia* cysts in wastewater samples prior to quantification by a real-time PCR method (data not shown). Moreover, this purification method could be used for the purification of *Giardia* cysts in sludge samples.

The present study provides, for the first time, information on the distribution of the genotypes of *G. lamblia* from humans with sporadic giardiasis in France. This work was based on a relatively small group of patients, but we are still pursuing analysis of sporadic giardiasis. However, our observation that the majority of sporadic giardiasis case isolates were assemblage B genotype (61.5%) corresponds to the findings of several studies conducted in India (100%, n = 10 [21]), Peru (76%, n = 25 [21]), United States (80%, n = 15 [11]), and United Kingdom (64%, n = 35 [2]). However, an Italian study reported 80% assemblage A in 30 stool samples examined by sequencing or PCR-RFLP analysis of the β-giardin gene (6). The predominance of assemblage B in samples collected in sewage treatment facilities was shown in one study (10). On the other hand, two other studies concluded that these was a majority of assemblage A in wastewater samples (6, 22). The use of RFLP analysis resulted in the same percentage for assemblage A group II and assemblage B group III, whereas assemblage B group IV was less common. The absence of assemblage A group I in these 26 fecal samples corresponds to the findings of the study conducted in the United Kingdom (2).

Among the 15 cases of giardiasis diagnosed in French sol-

diers coming back from the Ivory Coast, the detection of assemblage B group III in 14 confirmed the hypothesis of a common source of contamination. At present, only two studies have reported the genotyping results from outbreak-associated giardiasis. Among 24 samples from a nursery outbreak, Amar and colleagues (2) detected *G. duodenalis* assemblage B in 88% of the samples. Sulaiman and colleagues (21) detected assemblage B in two isolates from a food-borne outbreak by sequencing analysis of the *tpi* gene.

In our study, *G. lamblia* assemblage A and assemblage B were never detected together, whereas a mixture of these assemblages has been reported previously in a few studies (2, 11, 12, 14, 15). Thus, Amar and colleagues (2) observed a mixture of assemblage A group II and assemblage B in 9% of 35 samples, whereas Lu and colleagues (15) simultaneously detected assemblage A group I and assemblage B in 33% of only three samples.

In summary, the PCR assay developed in this study, combined with the ethyl acetate procedure, allowed rapid detection and genotyping of *G. lamblia* cysts from clinical samples. Our results show that the *tpi* gene is better adapted than the *gdh* gene for efficient discrimination between the two major assemblages. Thus, detection methods targeting loci with a high degree of polymorphism such as *tpi* can be extremely useful when a common source of contamination is certainly involved.

This work provides the first information about the distribution of the two major assemblages of *G. lamblia* in sporadic human giardiasis in France. However, further studies with a larger series of fecal or environmental samples could lead to better knowledge of the distribution of these assemblages in humans as well as the role of domestic animals and livestock as a potential source of infection for humans.

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